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Developmental Biology 289 (2006) 372 – 383

DEVELOPMENTAL BIOLOGY

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Brg1 is required for murine neural stem cell maintenance and gliogenesis

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Received for publication 10 August 2005, revised 4 October 2005, accepted 12 October 2005
Available online 2 December 2005

Abstract

Epigenetic alterations in cell-type-specific gene expression control the transition of neural stem cells (NSCs) from predominantly neurogenic to predominantly gliogenic phases of differentiation, but how this switch occurs is unclear. Here, we show that brahma-related gene 1 (Brg1), an ATP-dependent chromatin remodeling factor, is required for the repression of neuronal commitment and the maintenance of NSCs in a state that permits them to respond to gliogenic signals. Loss of Brg1 in NSCs in conditional *brg1* mutant mice results in precocious neuronal differentiation, such that cells in the ventricular zone differentiate into post-mitotic neurons before the onset of gliogenesis. As a result, there is a dramatic failure of astrocyte and oligodendrocyte differentiation in these animals. The ablation of *brg1* in gliogenic progenitors in vitro also prevents growth-factor-induced astrocyte differentiation. Furthermore, proteins implicated in the maintenance of stem cells, including Sox1, Pax6 and Musashi-1, are dramatically reduced in the ventricular zones of *brg1* mutant mice. We conclude that Brg1 is required to repress neuronal differentiation in NSCs as a means of permitting glial cell differentiation in response to gliogenic signals, suggesting that Brg1 regulates the switch from neurogenesis to gliogenesis.

Keywords: Neural stem cells; Brg1; Chromatin remodeling; Astrocytes; Neurons; Oligodendrocytes

Introduction

The major cell types comprising the mammalian central nervous system (CNS) arise from neural stem cells (NSCs), including neuroepithelial cells that transition into multi-potent radial glia, which initially reside in the ventricular zone (VZ) of the developing brain and spinal cord (Sauvageot and Stiles, 2002). Neurons, astrocytes and oligodendrocytes differentiate sequentially from NSCs, with most neurons being generated before glial cells. This pattern of differentiation is recapitulated in vitro, such that NSCs derived from early embryos (e.g., mouse embryonic day (E) 10–11) give rise exclusively to neurons after short-term culture, while cortical progenitors isolated after E13–14 become predominantly astrocytes under the same culture conditions (Qian et al., 2000). Furthermore, NSCs derived from early embryos switch from being predominantly neurogenic to predominantly gliogenic over time in vitro, suggesting that intrinsic changes in NSCs can regulate neuronal versus glial differentiation (Sun et al., 2003).

Epigenetic modifications to cell-type-specific genes contribute to the cell autonomous changes in NSCs that regulate neurogenic and gliogenic differentiation. The selective expression of transcription factors accompanied by chromatin remodeling, DNA methylation and localization of chromatin to specialized nuclear domains have all been implicated in regulating NSC fate determination (Hsieh and Gage, 2004; Cheng et al., 2005). Such alterations in cell-type-specific genes allow differentiated cells to maintain their identity even when confronted with extracellular cues that drive the differentiation of other cell types and are also required for NSC maintenance, ensuring that the needed numbers of cells are present to differentiate into neurons and glia at appropriate times during development. The signals that trigger these epigenetic changes are not well defined.

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doi:10.1016/j.ydbio.2005.10.044

We investigated the possibility that a subunit of SWI/SNF chromatin remodeling protein complexes, called brahma-related gene-1 (Brg1), is a key mediator that regulates neurogenesis versus gliogenesis. SWI/SNF chromatin remodeling complexes utilize energy from ATP hydrolysis to disrupt histone–DNA interactions, resulting in transcriptional activation or repression (Martens and Winston, 2003). Mammalian SWI/SNF complexes contain one of two catalytic ATPase subunits: Brm (for Brahma; also called SNF2α) and Brg1 (also called SNF2β). During mouse organogenesis, Brg1 transcripts are expressed in post-mitotic neurons and other cells in the mantle zone of the spinal cord and the intermediate and cortical layers of the forebrain in E12 mice, but less so in proliferating neurogenic NSCs in the VZ (Randazzo et al., 1994). By E15, there are increased levels of Brg1 transcripts in the predominantly gliogenic NSCs of the VZ and, later, in the subventricular zone (SVZ). Brg1 may therefore play a role in nervous system development. In support of this notion, zebrafish with *brg1* mutations have defects in terminal differentiation of retinal ganglion cells (Link et al., 2000; Gregg et al., 2003). Furthermore, in *Xenopus*, Brg1 is required for neuronal differentiation by mediating the transcriptional activities of the proneural bHLH proteins neurogenin-related-1 and NeuroD (Seo et al., 2005). Given that Brg1 is not expressed by predominantly neurogenic NSCs during early mouse development but is expressed later throughout the VZ during gliogenesis, we postulated that Brg1 signals the transition from neurogenesis to gliogenesis during mammalian development.

Materials and methods

Mice

Male nestin-cre mice were generously provided by Akira Imamoto, University of Chicago (Tronche et al., 1999) and were mated with female brg1$^{FL/FL}$ mice between 3 and 6 months of age. Animals were genotyped as previously described (Sumi-Ichinose et al., 1997).

Immunohistochemistry

Embryos were harvested, and CNS tissues were dissected and immersion-fixed at 4°C overnight, extensively washed in PBS and equilibrated in 30% sucrose. The tissue was embedded in freezing medium (TBS, Triangle Biomed. Sci.) and sectioned at 20 μm on a cryostat (Hacker Inst.). Sections were labeled with primary antibodies and detected with fluorescence-labeled secondary antibodies as previously described Liu (Strobeck et al., 2001; Potten et al., 2003; Liu et al., 2004a,b). Antibodies were obtained from the following sources: anti-Brg1 (Santa Cruz), HuC/D (Molecular Probes), cleaved-caspase-3 (Cell Signaling), GFAP (Dako), CD44 (ATCC), vimentin (Biomeda), RC2 (DSHB), Cre (Covance), PDGFRα (BD Pharmingen), Nkx2.2 (DSHB), Sox1 (DSHB), olig2 (kind gift of D. Rowitch), MBP (Chemicon), PLP (Chemicon), Ki67 (Novocastra), β-III tubulin (Covance), NeuN (Chemicon), S100β (Sigma) and Pax6 (DSHB). Anti-musashi-1 was a gift from Dr. H. Okano. DAPI was used at 1:5000 (Molecular Probes). TUNEL assays were performed using a DNA Fragmentation Assay kit (Clontech).

Cell culture

Cerebral cortices or GE were dissected from E12–14 Brg1$^{FL/FL}$ embryos. Tissue was incubated in trypsin–EDTA at 37°C for 10 min, washed and gently dissociated by trituration through a 25 g needle. Dissociated cells were grown for 1–5 days as "neurospheres" in a non-differentiating medium consisting of F12/DMEM (1:1; Gibco) supplemented with 0.1% BSA, B27, N2, FGF2 (a gift from F. Eckenstein; 10 ng/ml) and EGF (Upstate Biotech.; 10 ng/ml) and antibiotics. Neurospheres were dissociated and plated onto glass coverslips coated with poly-L-lysine and laminin (Sigma). Cultures were infected with 3.6×10^7 pfu of adeno-cre or adeno-EGFP (Gene Transfer Vector Core Facility, University of Iowa). Forty-eight hours after infection, some cultures were treated with CNTF (a gift from R. Nishi; 10 ng/ml) to induce astrocyte differentiation.

DNA microarrays

Total RNA from cortical and spinal tissues was isolated using TRIzol® (Invitrogen) following the manufacturer's instructions. Non-radioactive GEArray Q series cDNA expression array filters (Cat nos.: MM-0002, apoptosis; MM-001, cell cycle; MM-601.2, stem cells; SuperArray Inc.) were used to perform focused array analysis as described previously (Luo et al., 2005).

Results

Brg1 expression is upregulated in the mouse VZ after E13

Consistent with the localization of *brg1* transcripts (Randazzo et al., 1994), Brg1 protein was undetectable by immunohistochemistry in E13 and earlier mouse cortical and spinal cord VZ (Figs. 1a–e). Brg1 immunoreactivity was, however, detected throughout the cephalic neural tube and developing spinal cord by cells expressing the pan-neuronal marker, HuC/D, suggesting that neurons express Brg1 as they differentiate. Floor plate cells in the ventral spinal cord were also weakly positive for Brg1 (Fig. 1d, arrowhead). This pattern of Brg1 expression changes between E14 and birth, such that the majority of cells in the VZ as well as in the cortical and spinal cord gray matter become Brg1-immunoreactive (Figs. 1f–i). This change in Brg1 expression correlates with the transition of the cortical VZ from producing predominantly neuronal to predominantly glial cell progenitors. We therefore further investigated the role of Brg1 in producing these neural lineages.

Loss of Brg1 in the developing CNS causes severe cortical malformation

Because *brg1*-null mice die before implantation (Bultman et al., 2000), we tested the role of Brg1 in the developing murine CNS by crossing mice in which the *brg1* gene contains loxP sites in the intron upstream of exon 2 and in the intron downstream of exon 3 (Sumi-Ichinose et al., 1997) with mice expressing cre recombinase under the control of the nestin promoter (Tronche et al., 1999). The presence of *cre* and the deletion of *brg1* in nestin-cre (NC)-Brg1$^{FL/FL}$ mice were verified by PCR analysis of DNA from the brains of mutant as compared to wild type animals (Fig. 2a). By immunohistochemical analysis, NC-Brg1$^{FL/FL}$ mice lacked Brg1 protein in most regions of the CNS (Fig. 2b) and PNS (not shown). NC-Brg1$^{FL/FL}$ embryos developed at Mendelian ratios up to birth but died shortly thereafter, failing to inflate their lungs. Although the fetuses appeared grossly normal, brains from these mutant mice had severely malformed cerebral and cerebellar cortices, while the midbrain and brainstem appeared

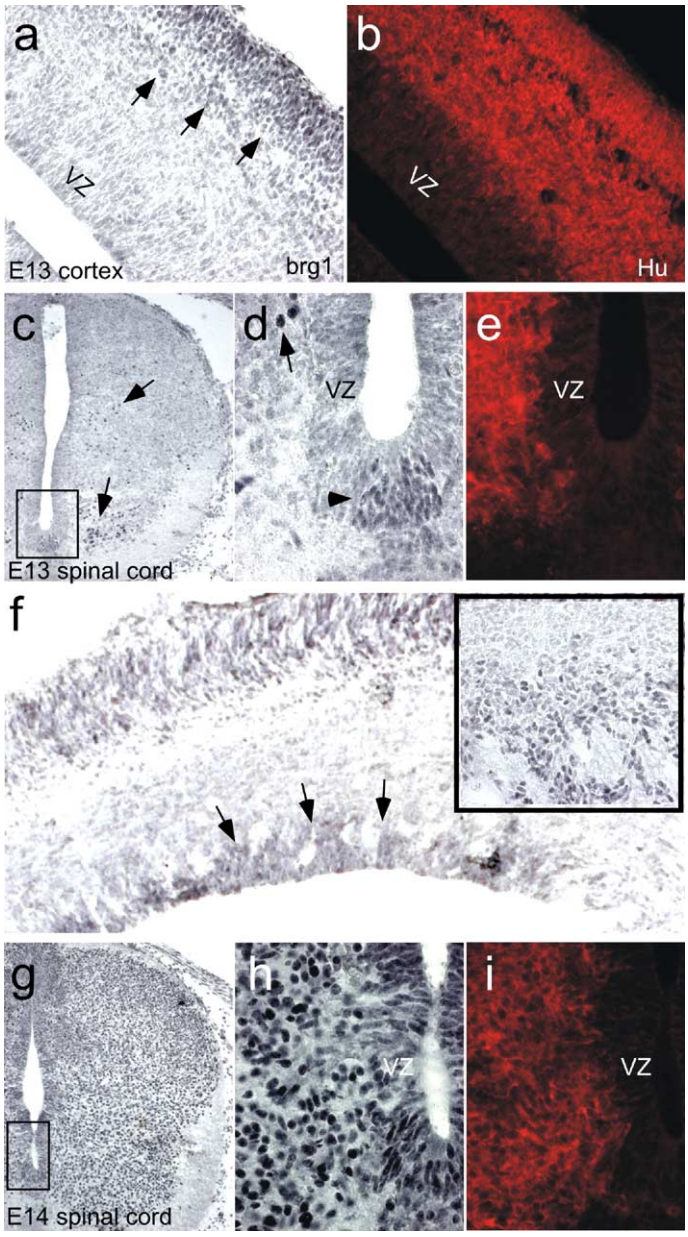

Fig. 1. Brg1 expression is induced in NSCs in the VZ after E13. Immunostaining for Brg1 (black; a, c, d, f, g, h) and HuC/D (red; b, e, i) in E13 (a–e) and E14 (f–i) cortex a, b, f and spinal cord c–e and g–i. Arrows in panel a indicate Brg1-labeled cells. Arrows in panels a, c and d indicate Brg1-labeled cells; arrowhead in panel d indicates floor plate cells in the developing spinal cord.

normal overall but diminished in size (Figs. 2c, d). Tissues outside of the CNS appeared normal (data not shown). A histological evaluation of the brains of E19 and older NC-Brg1$^{FL/FL}$ mice indicated that cerebral cortical organization was abnormal. In the oldest mutant mice examined (shortly after birth), we detected an extensive reduction in cell number and abnormal cytoarchitecture in the cerebral cortex (Figs. 2e–i and see below).

Early mouse neuronal differentiation does not depend on Brg1

To assess which specific cell types are affected by loss of Brg1, we labeled brain and spinal cord tissues from NC-Brg1$^{FL/FL}$ embryos with cell-type-specific markers. A screen with pan-neuronal markers indicated that neuronal differentiation proceeds apparently unabated at early stages (up to E13) in the cortex (Figs. 3a, b) and spinal cord (not shown) of NC-Brg1$^{FL/FL}$ mice. An examination of neuronal subtype-specific markers in *brg1* mutant mice indicated that the earliest generated cortical neurons, the Cajal–Retzius (CR) cells, begin to differentiate at their normal time (E10.5) and that they migrate, as expected, to the marginal zone where they form a plexus covering the cortical surface (Figs. 3c–h). These observations of early, outwardly normal neuronal differentiation in the absence of Brg1 are consistent with our finding that Brg1 is not detectable in the VZ during this stage of development.

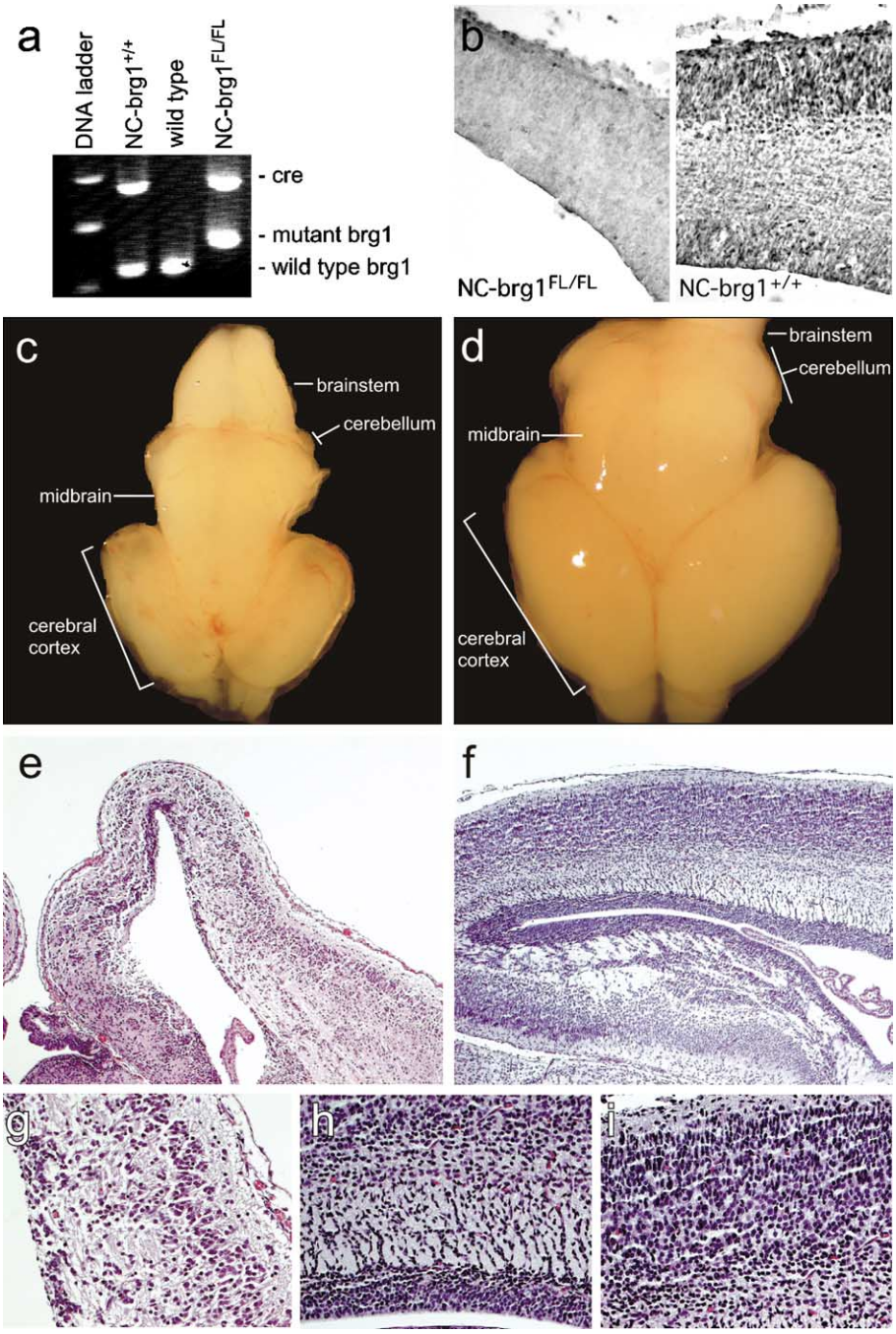

Fig. 2. Aberrant cortical development in the absence of Brg1. (a) PCR genotyping of embryos from female wild type and Brg1$^{FL/FL}$ mice mated with male nestin-cre (NC) mice. DNA from wild type mice served as a negative control. (b) Sections of E15.5 cerebral cortex labeled with an anti-Brg1 antibody verifying the loss of Brg1 expression in NC-*brg1*$^{FL/FL}$ mice. (c) Whole brain from a neonatal NC-*brg1*$^{FL/FL}$ mouse and (d) a wild type littermate. Both brains were imaged at the same magnification. (e, g) Hematoxylin and eosin-stained paraffin sections of neonatal NC-*brg1*$^{FL/FL}$ and (f, h, i) wild type littermate cerebral cortex. Note the reductions in cortical thickness and cell density in the mutant.

Loss of Brg1 influences neuronal phenotypes after NSCs become committed to neuronal lineages

Although the brg1 mutant CR neurons display an outwardly normal pattern of differentiation at early stages, there are two potentially revealing anomalies that become evident once neurons begin to express early neuronal markers (e.g., HuC/D). First, CR neurons form an abnormally thick layer of cells in the mutant cortex (compare Figs. 3c and d). This may be the result of a relatively normal complement of cells populating the progressively reduced surface area of the mutant cortex. Second, there is evidence that the CR cells are maturing precociously. We observe, for example, calretinin^{+}/reelin^{+} cells in the ventricular and developing intermediate zones of the brg1 mutant embryos (compare Figs. 3e, f and g, h). These cells appear to be CR cells in the process of migrating to the cortical surface. Control CR cells do not normally display calretinin or reelin immunoreactivity until after they have completed migration (e.g., Figs. 3e, f).

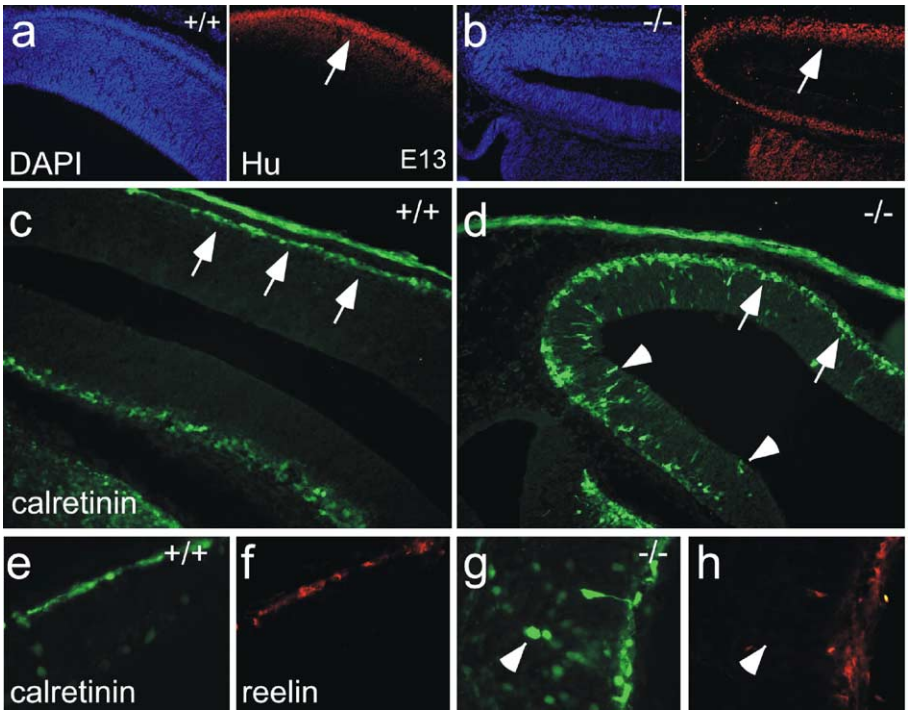

Fig. 3. Premature loss of the VZ in NC-*brg1*$^{FL/FL}$ mice. (a, b) Sections from E13 cerebral cortex of wild type (+/+; a) and NC-*brg1*$^{FL/FL}$ (−/−; b) mice labeled with DAPI (blue) or HuC/D (red). Note that neurogenesis, including cortical plate development (arrows), appears essentially normal in the mutant cortex. (c, d) Calretinin immunolabeling of cortical sections from E12 wild type (c) and NC-*brg1*$^{FL/FL}$ (d) mice. Note that CR neurons form at the normal time and migrate to the marginal zone (arrows), but they express calretinin before migrating from the VZ (d, arrowheads). (e–h) Calretinin (green) and reelin (red) immunolabeling in cortical sections from E14 mice. Calretinin^{+}/reelin^{-} (i.e. not CR neurons; arrowheads) cells are located in the developing intermediate zone of mutant mice.

Additional deficits in cortical neuronal development are evident after E14, when there are abnormalities in cortical layering and pyramidal neuron morphology in the NC-Brg1$^{FL/FL}$ mutants (e.g., compare Figs. 4a and b). Furthermore, after E14, nearly all of the cells in the VZ appear to be post-mitotic neurons as they are Ki67^{-} and HuC/D^{+} (compare Figs. 4c and d, and e and f). At the oldest stages examined (between E18 and immediately following birth), we observed a dramatic loss of neurons in the cortex (e.g., Fig. 4b). This loss is likely due, in part, to a deficiency in trophic support as a result of the loss of glia (see below). However, given that the VZ becomes filled with post-mitotic HuC/D^{+} cells by E14, it is likely that many neuronal progenitors are also exhausted before E14, resulting in the failure of later neuronal populations to differentiate in the absence of Brg1 (see below).

Cortical neurons derived from subcortical proliferative zones are also affected by the absence of Brg1. Many cortical interneurons, for example, migrate along a tangential pathway into the cortex from their origins in the ganglionic eminences (Lavdas et al., 1999). Normally, the location of these cells during their migration cannot be determined without experimental intervention (injecting a dye or forcing the expression of a marker). In the NC-Brg1$^{FL/FL}$ mutant cortex, however, large numbers of calretinin^{+} cells are observed throughout the migratory pathway of these interneurons (compare Figs. 4g and h). These calretinin^{+} neurons can be distinguished from the CR cells because they are not reelin^{+} (e.g., Figs. 3g, h). At later stages, the organization of the calretinin^{+} neurons in the deeper cortical layers is grossly abnormal in the mutants, including a failure to form the subplate at E15 (not shown).

These data suggest that Brg1 regulates neuronal maturation but that, unlike in lower vertebrates (Seo et al., 2005), it is not necessary for the initiation of neuronal differentiation in mammals. To test if Brg1 regulates neuronal maturation, we cultured neurogenic NSCs from E12 ganglionic eminences (GE) of Brg1$^{FL/FL}$ mice. Brg1 expression was abolished by infecting the cultures with an adenovirus carrying a cre recombinase expression vector (adeno-cre; Figs. 4i–k). Controls were infected with an adenovirus containing an enhanced green fluorescent (adeno-EGFP) expression vector. Typically, >90% of the cells were infected by the adenovirus (e.g., Figs. 4i, j). Adeno-cre-infected NSCs lost Brg1 expression within 48 h post-infection (Fig. 4k). Loss of Brg1 from GE-NSCs caused a rapid (within 24 h of viral infection) increase in the numbers of cells expressing calretinin (in one experiment, the percentage of calretinin^{+} cells increased from 0.8% in control cultures to 17% in adeno-cre-treated cultures without a significant change in total cell number; see Figs. 4l, m). These cells became post-mitotic as indicated by loss of Ki67 labeling and a lack of BrdU incorporation (data not shown). These data suggest that Brg1 regulates neuronal differentiation.

Most macroglia fail to differentiate in brg1 mutant mice

The loss of Brg1 had profound effects on gliogenesis. The NC-Brg1$^{FL/FL}$ mice displayed a dramatic reduction in the

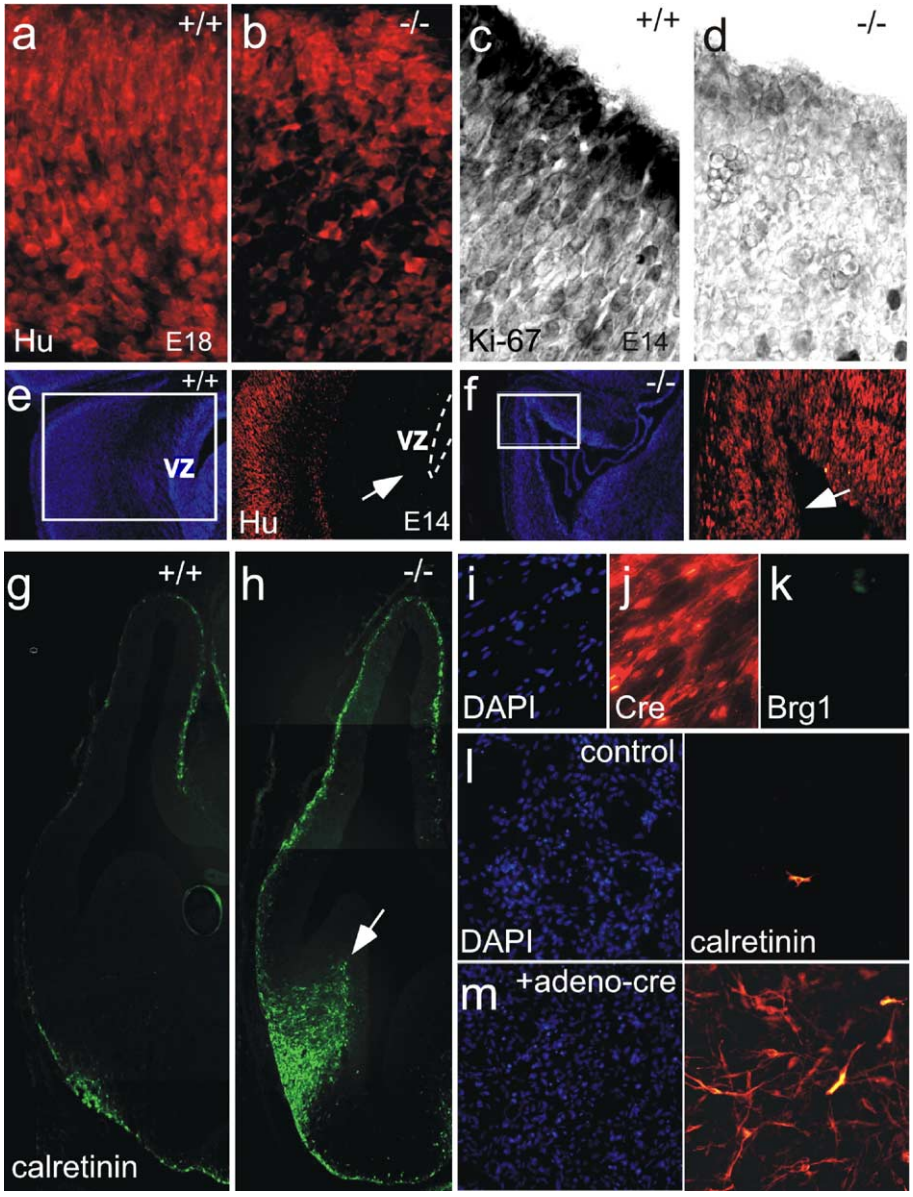

Fig. 4. Premature maturation of cortical neurons in NC-*brg1*$^{FL/FL}$ mice. (a, b) HuC/D labeling of cortical sections from wild type (a) and mutant (b) cortex at E18. Note that, in the absence of Brg1, both cytoarchitecture and neuronal morphology are aberrant. In particular, cells with the morphologies of pyramidal neurons do not appear to be present in the mutants. (c, d) Ki-67 labeling in the VZ of E14 wild type (c) and mutant (d) mice. By this stage, nearly all of the cells in the VZ appear to be post-mitotic in NC-*brg1*$^{FL/FL}$ mice. (e, f) HuC/D and DAPI labeling of wild type (e) and mutant (f) cortex at E14. In wild type mice, there is a clear VZ that lacks HuC/D immunoreactivity (arrow). In NC-*brg1*$^{FL/FL}$ mice, however, the area where the VZ should be is comprised of HuC/D^{+} cells. (g, h) Calretinin immunolabeling in the GE of wild type (g) and mutant (h) mice. Note the extensive calretinin labeling in the GE of mutant mice (h, arrow). (i–k) Cultures of NSCs derived from an E12 *Brg1*$^{FL/FL}$ mouse and infected with an adenovirus carrying a cre recombinase expression vector (adeno-cre). Infection efficiency was >90% as demonstrated in panel j where virtually all DAPI-positive cells (i) express cre and show no Brg1 immunoreactivity (k). (l, m) Calretinin (red) and DAPI (blue) labeling in GE-NSCs cultured from E12 *Brg1*$^{FL/FL}$ mice and infected with a control adenovirus or adeno-cre. Note that, in the absence of Brg1, NSCs express calretinin (m).

numbers of cells expressing CD44 and S100β, markers of astrocyte progenitors, and GFAP, a marker of mature astrocytes, in the brains and spinal cords of *brg1* mutant mice (Figs. 5a–h) between E14 and E18. This finding suggests that cells in the astrocyte lineage were absent from most regions of the CNS in these animals. Consistent with this idea, cortical sections from *brg1* mutant mice displayed extensive HuC/D immunoreactivity in areas of presumptive white matter (e.g., Fig. 4f) and, when stained with hematoxylin and eosin, very few cells with astrocyte-like morphologies (data not shown). The proportion of cells in the mutant cortex and spinal cord that expressed oligodendrocyte progenitor markers (olig2, nkx2.2, PDGFRα) as well as myelin protein markers (PLP and MBP) was also dramatically reduced between E14 and E17 (Figs. 5i, j and data not shown). These findings are consistent with the idea that Brg1 is required for the differentiation and/or maturation of astrocytes and oligodendrocytes.

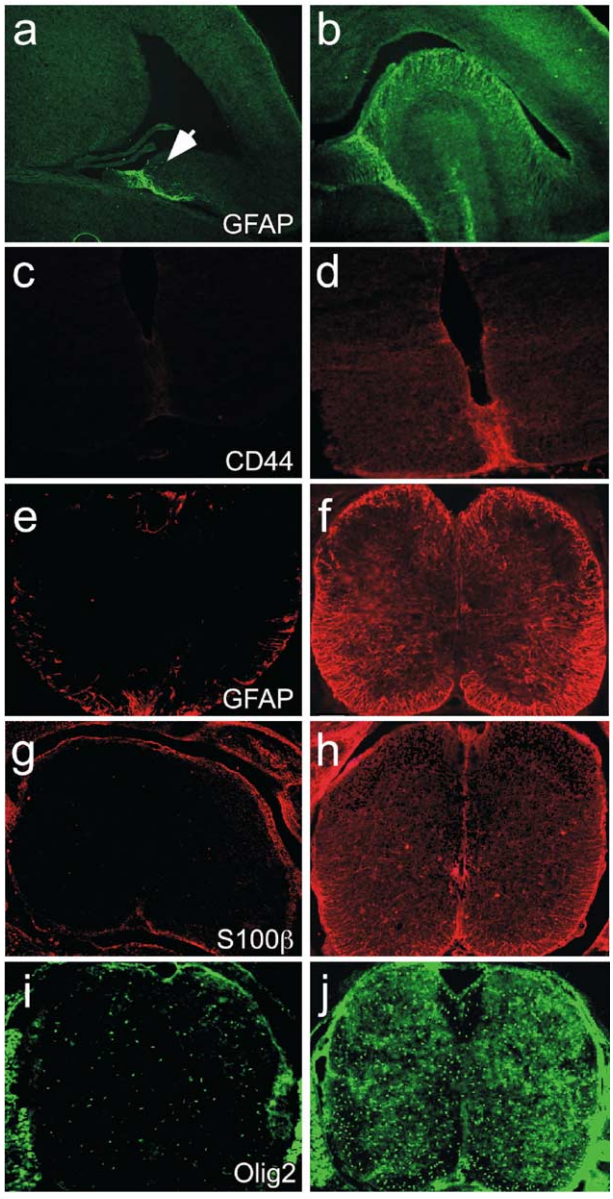

Fig. 5. Reduced glial cell differentiation in the absence of Brg1. (a, b) Frontal sections of cerebral cortex from an E18.5 NC-Brg1$^{FL/FL}$ mouse (a) and wild type littermate (b), showing a dramatic loss of GFAP immunoreactivity in the mutant brain, with the exception of limited subcortical areas (e.g., arrow). (c–j) Spinal sections from E14 mutant (left panels) and wild type (right panels) mice labeled with antibodies against astrocyte markers, including CD44 (c, d; an early marker of astrocyte differentiation (Liu et al., 2004a,b)), GFAP (e, f) and S100β (g, h) as well as the early oligodendrocyte marker olig2 (i, j). Note that all markers of glial progenitor cells are dramatically reduced in NC-*brg1*$^{FL/FL}$ mice.

Brg1 is required for astrocyte differentiation

To determine if Brg1 directly influenced gliogenesis, we examined the ability of gliogenic cortical NSCs harvested at E14 to differentiate into astrocytes in the presence or absence of Brg1. Cortical NSCs from E14 Brg1$^{FL/FL}$ mice were placed into cell culture and infected with either adeno-cre or adeno-EGFP as above. The cultures were induced to differentiate into astrocytes by adding ciliary neurotrophic factor (CNTF). If CNTF was added 48 h after infection with adeno-cre (when Brg1 expression was ablated; Figs. 6a, b insets), GFAP-labeled cells with astrocyte-like morphologies were not detected (compare Figs. 6a and b). Instead, all of the GFAP$^+$ cells in these cultures had very long, unbranched processes that were also vimentin$^+$, suggesting that they were radial glial cells (Figs. 6c, d). We did not observe any differences in the levels of cell death in control as compared to adeno-cre-infected cultures as assessed by TUNEL labeling and immunostaining with an anti-cleaved caspase-3 antibody, and Brg1 did not influence BrdU incorporation (data not shown). If adeno-cre was added at the same time as CNTF or later, numerous GFAP$^+$ cells with astrocyte-like morphologies arose in these cultures (not shown), suggesting that Brg1 is not required for astrocyte survival once astrocyte differentiation has been induced.

Brg1 is required to maintain gliogenic populations of NSCs

We conclude from these studies that Brg1 is not required for neuronal differentiation but is required for aspects of neuronal maturation and for the differentiation of gliogenic NSCs into astrocytes and at least some oligodendrocytes. Reduced numbers of glial cells in NC-Brg1$^{FL/FL}$ mice may result from the depletion of NSCs from the VZ during the neurogenic period. In support of this hypothesis, we observed a decrease in radial glia during the neurogenic to gliogenic transition in *brg1* mutant animals. Vimentin$^+$/RC2$^+$ radial glia were present in the Brg1 mutant mice but in reduced numbers when examined in brains and spinal cords at E14 and E17

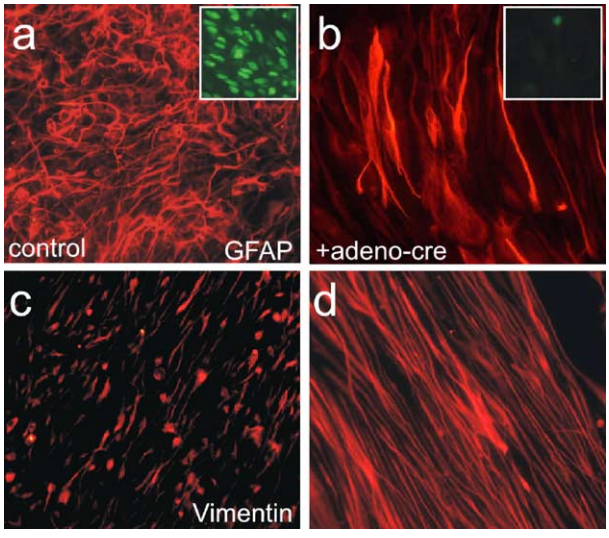

Fig. 6. Loss of Brg1 directly inhibits astrocyte differentiation by gliogenic NSCs in vitro. (a–c) Cultures of cortical NSCs derived from E14 NC-*brg1*$^{FL/FL}$ mice infected with adeno-cre (right panels) or a control adenovirus (left panels) and labeled with either an anti-GFAP (a, b) or an anti-vimentin (c, d) antibody. Cells were treated with CNTF (10 ng/ml) following infection to promote astrocyte differentiation. Note the presence of GFAP-immunoreactive cells with astrocyte morphology in the control cultures but only radial glial-like cells in the adeno-cre-infected cultures. Insets in panels a and b show that Brg1 immunoreactivity is ubiquitous in control cultures and virtually absent from adeno-cre-infected cultures.

(Figs. 7a–f). To test if stem cell marker expression was generally altered in the Brg1 mutant mice, we analyzed the expression of a large number of stem-cell-related genes by DNA microarray analysis. A small number of stem-cell-related genes were expressed at lower levels in Brg1 mutant mice at E14 as compared to wild type animals (Table 1). Antibody screens for the expression of some of these markers confirmed that NSC numbers were dramatically reduced in the brains and spinal cords of Brg1 mutant mice, including Sox1 (Figs. 7g–j) and Pax6 (Figs. 7k–n).

These data suggest that Brg1 is required to maintain the expression of at least some NSC-related genes or, possibly, for the maintenance of NSCs themselves. We tested this idea further by labeling sections of wild type and NC-Brg1$^{FL/FL}$ cortical sections at E10, E11, E14 and E17 for Musashi-1, an RNA-binding factor that inhibits NSC differentiation (Kaneko et al., 2000; Okano et al., 2002; Sakakibara et al., 2002). We could not detect any differences in Musashi-1 immunoreactivity up to E11 (Figs. 8a, b). However, Musashi-1 was significantly reduced in *brg1* mutant cerebral cortex by E14 (Figs. 8c–h) and virtually absent by E17 (Figs. 8i–n).

As discussed above, the VZ appeared to be absent or severely reduced in size throughout the CNS in NC-Brg1$^{FL/FL}$ mice by E14. Ventricular layers in the NC-Brg1$^{FL/FL}$ brain were populated with cells expressing neuronal markers, including HuC/D (e.g., Fig. 3h) and beta-III tubulin (not shown). The area surrounding the central canal of the spinal cord was similarly filled with cells expressing markers expressed by post-mitotic neurons in *brg1* mutant mice (Fig. 9). These data, along with the reduction of NSC markers described above, support the hypothesis that the loss of Brg1

Table 1
Altered expression of NSC-related transcripts in Brg1 mutant mice

Genebank	Symbol	Gene name	Fold change over wt
NM_008634	MAP1B	Microtubule-associated protein 1B	1.3
Y00051	NCAM1	Neural Cell Adhesion Molecule 1	0.4
U46155	Ptch	Patched (*Drosophila*) homolog	0.3
NM_009819	Catna2	Catenin alpha 2	0.3
M27130	CD44	CD44 glycoprotein	−0.01
NM_009233	Sox1	SRY-box containing gene 1	−0.1
AJ010604	Sox5	SRY-box containing gene 5	−1.3
NM_013627	Pax6	Paired box gene 6	−4.8
NM_008809	Pdgfrb	PDGF receptor, beta	−16.8

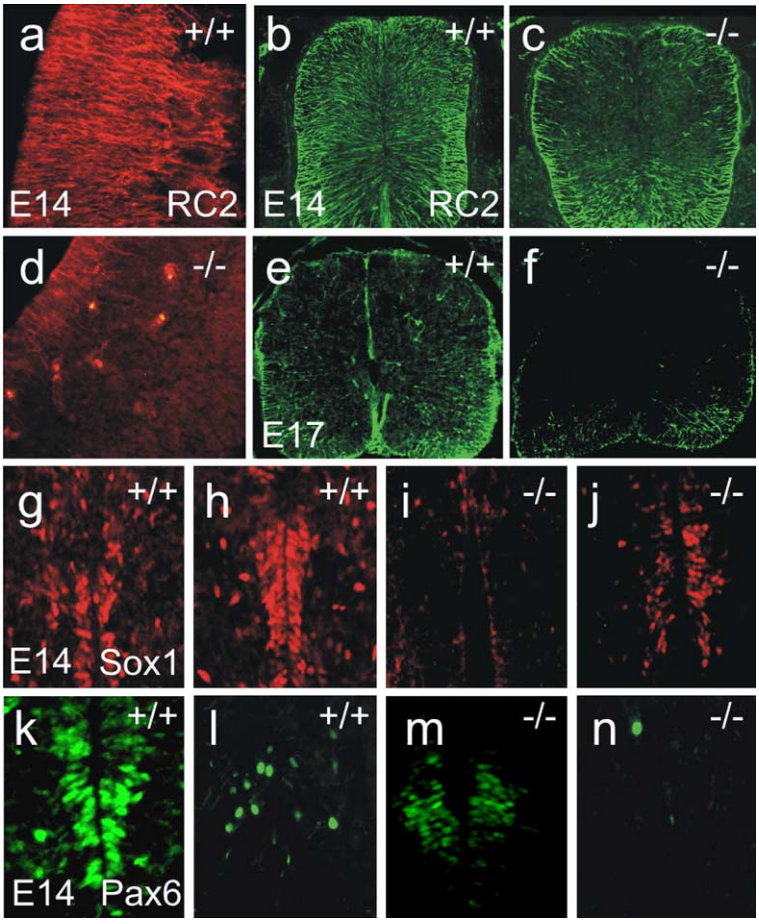

Fig. 7. Loss of Brg1 results in reduced numbers of NSCs in the VZ of mice during gliogenesis. (a–f) RC2^{+} radial glia are reduced in the VZ of cortex (a, d) and spinal cord (b, c, e, f) of NC-*brg1*$^{FL/FL}$ mice at E14 (a–d) and throughout spinal tissues by E17 (e, f). (g–i) Sox1 expression in E14 dorsal (g, i) and ventral (h, j) spinal cord VZ from wild type and *brg1* mutant mice. (k–n) Pax6 expression in E14 ventral (k, m) spinal cord VZ and adjacent mantle zone (l, n) from wild type and *brg1* mutant mice.

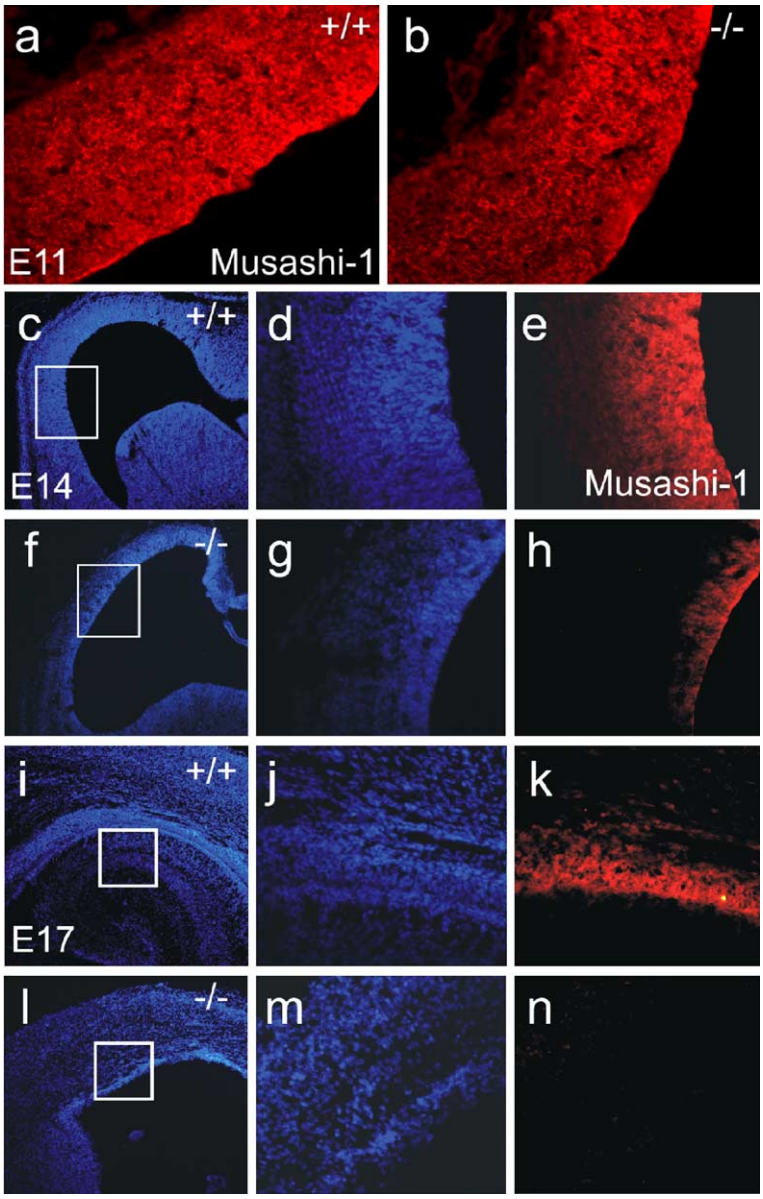

Fig. 8. Musashi-1 expression is dramatically reduced in NC-*brg1*$^{FL/FL}$ NSCs. Expression of Musashi-1 (a, b, e, h, k and n; red) in sections from E11 (a, b). E14 (c–h) and E17 (i–n) wild type and *brg1* mutant mice double labeled with DAPI (blue). Panels in d, e, g, h, j, k and m, n are enlargements of the boxed areas to the left. Note that musashi-1 expression appears to be unaltered at E11 but is dramatically decreased after E14.

leads to a reduction in the number of NSCs that can differentiate into astrocytes and oligodendrocytes. This effect could be linked to altered cell cycle progression, leading to premature neuronal differentiation, apoptosis of gliogenic progenitors or both. Indeed, interactions between Brg1 and the retinoblastoma tumor suppressor (pRB) can regulate cell cycle progression by repressing targets of the E2F transcription factor family, including cyclin E, cyclin A and CDC2 (Dunaief et al., 1994; Strober et al., 1996; Trouche et al., 1997; Strobeck et al., 2000; Zhang et al., 2000) and loss of Brg1 in other cell types can result in E2F-mediated hyperproliferation (Dahiya et al., 2000) or apoptosis (Liu et al., 2004a,b). We tested whether Brg1 influenced the expression of genes that regulate cell cycle progression using a targeted DNA microarray. By E14, the levels of numerous cell cycle gene transcripts were altered in a pattern consistent with cell cycle arrest (Table 2). In agreement with these data, Ki67 (Figs. 4c, d) and PCNA (not shown), markers for proliferating cells, were severely reduced in the pallial VZ of E13.5 and older cortices but not at earlier stages. A sample count of Ki67-labeled nuclei in the cortical ventricle zone indicates very few proliferating cells at E14 (4 ± 3%) versus controls (57 ± 18%).

Loss of proliferating cells in the VZ could be the result of NSCs undergoing apoptosis or differentiating into post-mitotic cells. Although we did not observe a significant increase in pro-apoptotic gene expression at E14 in Brg1 mutant brains (Table 3), we did observe increased labeling of the pro-apoptotic marker cleaved caspase-3 in some VZ cells beginning at E13 (Figs. 10a–e). These cells did not express RC2, indicating that the predominant gliogenic NSC population was

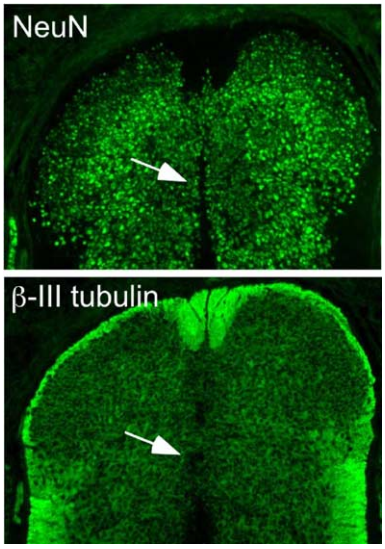

Fig. 9. Aberrant expression of neuronal markers in *brg1* mutant spinal cord. Spinal cord sections from E14 *brg1* mutant mouse spinal cords, showing NeuN (top panel) and β-III tubulin (bottom panel) staining in the VZ (arrows).

not dying (Fig. 10e). Instead, the cells appeared to have neuronal morphologies (e.g., Figs. 10f–i), and many of these cells co-labeled with HuC/D (data not shown), indicating that they were differentiated neurons. These findings are consistent with our in vitro data, where we did not observe altered levels of cell death in cells lacking Brg1. Apoptosis alone cannot, therefore, explain the failure of Brg1-null NSCs to undergo gliogenesis. It is possible that the few cells undergoing apoptosis are dying due to lack of trophic signals.

Discussion

Our data indicate that Brg1 is a critical regulator of NSC differentiation. Brg1 represses neuronal commitment in NSCs prior to the onset of gliogenesis, permitting NSCs to remain undifferentiated until they respond to gliogenic signals. This function of Brg1 is distinct from its role in lower vertebrates. In *Xenopus*, for example, virtually all neuronal differentiation was blocked by inhibiting the expression of Brg1 or by blocking its function (Seo et al., 2005). In zebrafish, the effect of ablating *brg1* was restricted to a loss of retinal ganglion cells (Gregg et al., 2003). In *Xenopus*, the loss of Brg1 appears to cause a failure of cell cycle arrest and an expansion of NSCs. Here, we demonstrate a completely different role for Brg1 in mammalian neural development. A targeted deletion of *brg1* in mouse NSCs leaves the early generation of neurons intact, while the

Table 2
Altered expression of cell cycle-related transcripts in Brg1 mutant mice

Genebank	Symbol	Gene name	Fold change over wt
U36475	Brca1	Breast cancer 1	0.3
AF132483	cdk6	cyclin-dependent kinase 6	−2.0
AF287135	Ccnh	Cyclin H	−2.4
X58708	Ccnb1-rs1	Cyclin B	−2.5
AF016583	Chek1	Checkpoint kinase Chk1	−3.0
Z47766	Ccnf	Cyclin F	−4.5
L76150	Cdkn2a	p16	−5.5
AF032131	E2F-6	E2F-like transcriptional repressor	−5.6
M64403	Ccnd1	Cyclin D1	−5.8
U62638	Ccnc	Cyclin C	−6.0

Table 3
Altered expression of apoptosis-related transcripts in Brg1 mutant mice

Genebank	Symbol	Gene name	Fold change over wt
U19522	casp3	caspase 3	0.5
AJ224738	Cradd	Death adaptor molecule	0.5
NM_009811	casp6	caspase 6	0.4
AF027707	Bok/mtd	Bcl-2-related ovarian killer protein	0.4
D83698	Bid3	BH3 interacting (with BCL2 family)	0.2

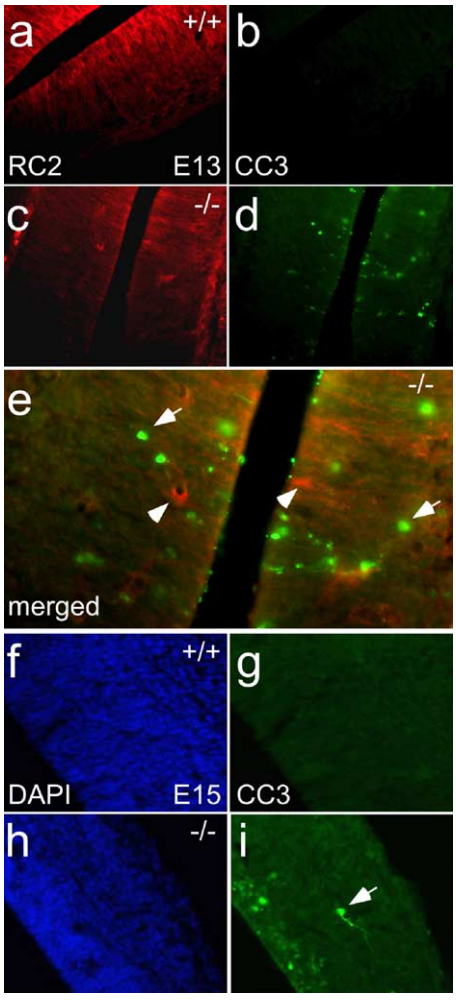

Fig. 10. Increased expression of cleaved caspase-3 in the cortical VZ of NC-*brg1*$^{FL/FL}$ mice. (a–e) Sections from E13 wild type (+/+) and *brg1* mutant (−/−) mice labeled with RC2 (red) or cleaved caspase-3 (CC3; green). As seen in the merged image (e), RC2^{+} radial glia (e.g., arrowheads) are not immunolabeled with CC3 (e.g., arrows). Given that the majority of the CC3^{+} cells have large cell bodies and that nearly all of the cells in VZ are HuC/D^{+} in the mutant mice, the few cells that are undergoing apoptosis are likely to be post-mitotic neurons. (f–i) DAPI (blue) and CC3 (green) labeling in E15 wild type and *brg1* mutant mice. Note that there are still some dying cells with neuronal morphologies (e.g., arrow) at this stage of development.

subsequent generation of glia is virtually abolished. Also, unlike *Xenopus*, NSCs in the mouse VZ are depleted at the expense of precocious neuronal differentiation instead of being expanded in the absence of Brg1. In addition, we find that the in vitro differentiation of late gliogenic NSC into neurons is unaffected by the loss of Brg1, but astrocyte differentiation is completely blocked. The striking contrast between our findings in the mouse compared to the previous studies in non-amniotic vertebrates suggests extensive divergence in the role of chromatin remodeling factors in mediating neural development.

The phenotype of the NC-Brg1$^{Fl/FL}$ mice and the cell culture experiments suggest that Brg1 is a critical mediator of several stages of mammalian neural development. We propose that Brg1 suppresses neuronal differentiation in gliogenic NSCs and mediates aspects of neuronal maturation after cells have become committed to neuronal fates. It is clear that the early generation of neurons, including the earliest born CR neurons, occurs unperturbed in the *brg1* mutant mice. Combined with the observation that Brg1 expression does not appear in the VZ until the onset of gliogenesis, our data suggest that Brg1 is not required for the initial differentiation of neurons. Early stages of neuronal maturation, however, do appear to be abnormal in the *brg1* knockouts, consistent with the observation that neurons express Brg1 once that they have begun to differentiate and migrate away from the VZ. The timing of calretinin expression is premature in the mutant CR neurons and in a subpopulation of cortical interneurons. Brg1 may, therefore, play a critical role in defining neuronal subtype identities once cells have become committed to neuronal differentiation.

One way that Brg1 may influence neuronal subtype specification is through interactions with the RE1 silencing transcription factor, REST. REST is a zinc finger protein that binds to a conserved 23 bp motif called RE1 (Chong et al., 1995; Schoenherr and Anderson, 1995). As NSCs differentiate into neurons, REST and its co-repressors dissociate from RE1 sites, allowing for the transcription of neuron-specific genes. In some genes, however, transcription is regulated through interactions with methylated DNA sequences that are distinct from RE1 (Ballas et al., 2005). SWI/SNF complexes that contain Brg1 can interact with REST repressor complexes, and antibodies against Brg1 can relieve the repression of RE1 reporter genes (Battaglioli et al., 2002). Furthermore, recent evidence indicates that Brg1 can influence the transcription of some genes by altering DNA methylation (Banine et al., 2005). It is possible, therefore, that, as Brg1 becomes expressed in gliogenic NSCs, it interacts with REST complexes to prevent neuronal differentiation. It may also, however, interact with similar complexes in differentiating neurons to define neuronal subtypes.

It is unclear if Brg1 is also required for glial cell differentiation. Our data suggest that, in the absence of Brg1, gliogenic NSCs either become neurons or radial glia. One possible way that Brg1 could regulate gliogenesis is through interactions with the nuclear receptor co-repressor, N-CoR, which actively represses astrocyte differentiation (Hermanson et al., 2002). Brg1 and other SWI/SNF proteins interact with N-CoR (Underhill et al., 2000), suggesting that the loss of Brg1 could alter N-CoR function in cells that would normally differentiate into astrocytes. It is unclear, however, whether Brg1 inhibits or promotes N-CoR-mediated transcriptional repression. Nonetheless, our results have significant implications for understanding how NSC fates are regulated at different times during mammalian embryonic development and may provide clues about how NSC differentiation can be manipulated in a variety of pathological conditions.

Acknowledgments

We thank H. Okano for the musashi-1 antibody. This work was supported in part by NIH grant NS39550 to L.S., a grant from the Medical Research Foundation of Oregon to F.B., a grant from the Christopher Reeve Paralysis Foundation (SB1-0510-2) to L.S. and NIH funds supporting the Oregon National Primate Research Center (RR00163).

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